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## Targeting the microenvironment of pancreatic cancer: overcoming treatment barriers and improving local immune responses

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### Abstract

Historically, patients diagnosed with metastatic pancreatic cancer have faced a grim prognosis. The survival benefit seen with systemic chemotherapies and even combinations thereof have been disappointing. However, growing data suggest that the microenvironment of pancreatic cancer may be contributing to this poor prognosis. This microenvironment has a dense fibrotic stroma, and is hypoxic and highly immunosuppressive, all of which pose barriers to treatment. Newer strategies looking to disrupt the fibrotic stroma, target hypoxic areas, and improve local immune responses in the tumor microenvironment are currently undergoing clinical evaluation and seem to offer great promise. In addition to these therapies, preclinical work evaluating novel cytotoxic agents including nanoparticles has also been encouraging. While much research still needs to be done, these strategies offer new hope for patients with pancreatic cancer.

### Keywords

Pancreatic cancer; Microenvironment; Immune responses

### Introduction

Pancreatic cancer is the twelfth most common cancer type among adults in the United States and approximately 1.5 % of men and women will be diagnosed with the disease in their lifetime [1], Despite its low incidence, it carries a high mortality rate and is the fourth leading cause of cancer death in the US. It is predicted that 40,560 adults will die from pancreatic cancer in the US in 2015 [2], This number is not far off from the estimated mortality of breast (40,730) and colorectal cancer (49,700) in 2015 and it is projected that

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Compliance with ethical standards

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the mortality rate of pancreatic cancer will eclipse the rates of both these cancers in the next few years.

The high mortality rate is likely due to a number of factors. Firstly, because of the location of these tumors, there is high propensity for negative sequelae including gastric outlet as well as biliary obstruction and peritoneal carcinomatosis. Secondly, tumors which arise in the body and tail of the pancreas are often asymptomatic early on, and the vast majority of patients with these tumors often have metastases at the time of diagnoses [3]. Thirdly, the currently available therapies for this disease offer only modest improvements in overall survival. Historically, the 5-year survival rate for patients with metastatic disease has been less than 3 % and even for patients diagnosed with localized disease the 5-year survival rate has reportedly ranged between 19 and 27 % [1, 4]. Until 2011, there were a few standard of care treatments for patients with metastatic pancreatic cancer which included single-agent gemcitabine [5], gemcitabine plus erlotinib [6], and combination chemotherapy with gemcitabine plus either a platinum or fluoropyrimidine-based agent [7].

More recently, two trials evaluating combination chemotherapy in well-functioning patients with metastatic pancreatic cancer have shown improved outcomes compared to gemcitabine monotherapy. In the first trial, 342 patients with metastatic pancreatic cancer were randomized to FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin) every 2 weeks or gemcitabine weekly for 7 of 8 weeks followed by weekly for 3 of 4 weeks. The median overall survival of patients receiving FOLFIRINOX was significantly better than patients receiving gemcitabine (11.1 vs. 6.8 months; HR 0.57; 95% CI 0.45–0.73;  $P < 0.001$ ) [8]. In the second trial, 861 patients with metastatic pancreatic cancer were randomized to nab-paclitaxel followed by gemcitabine weekly for 3 of 4 weeks or gemcitabine weekly for 7 of 8 weeks followed by weekly for 3 of 4 weeks. The median overall survival of patients receiving nab-paclitaxel followed by gemcitabine was also significantly better than patients receiving gemcitabine alone (8.5 vs. 6.7; HR 0.72; 95 % CI 0.62–0.83;  $P < 0.001$ ) [9].

Many other chemotherapy regimens have been tested and found ineffective for locally advanced and metastatic pancreatic cancer. Increasing evidence suggests that the highly fibrotic, hypoxic, and immunosuppressed microenvironment of this disease may be contributing to this lack of response to therapy and overall poor prognosis. In response, there is growing optimism over newer agents looking to target these areas. This review will summarize early data evaluating these newer agents aimed at the microenvironment of pancreatic cancer.

## Stromal disrupting approaches

The microenvironment of pancreatic cancers is composed of a highly desmoplastic and fibrotic stroma consisting of among other extracellular components stellate cells (myofibroblasts), type I collagen, and hyaluronic acid. Components of this stroma including pancreatic stellate cells have been found to support tumor cell growth, migration, and invasion [10]. Type I collagen, connective tissue growth factor (CTGF) as well as other factors produced in this stroma have been found to contribute to chemotherapy resistance [11, 12]. In addition, this dense stroma is further characterized by high interstitial fluid

pressures, poor vascular perfusion, and hypoxia [13] which may further hinder drug delivery. In preclinical mouse models of pancreatic cancer, agents enzymatically targeting hyaluronic acid have produced decreased interstitial pressure, increased vascular patency, and improved responses in combination with chemotherapy [14]. Based upon this work, two early-phase trials are now evaluating pegylated recombinant human hyaluronidase (PEGPH20) in combination with standard chemotherapy (NCT01839487, NCT01959139) in patients with newly diagnosed metastatic pancreatic cancer. Interim results from the first of these two trials were promising. In this phase II trial, patients with untreated metastatic pancreatic cancer were randomized to gemcitabine plus Nab-paclitaxel with or without PEGPH20. 135 patients were randomized. Forty-eight of these patients had tumors which were reported to have a high hyaluronic acid burden and this subgroup of patients appeared to benefit significantly from the addition of PEGPH20. Progression-free survival (PFS) in this group was increased from 4.3 to 9.2 months with the addition of PEGPH20 ( $P = 0.03$ ) [13]. A number of additional approaches targeting tumor stroma are currently under investigation. Two early-phase trials are evaluating FG-3019, an antibody against CTGF, in combination with standard chemotherapy for patients with locally advanced [NCT02210559] and metastatic pancreatic cancer [NCT01181245].

## Overcoming the hypoxic microenvironment

The finding that the pancreatic tumor microenvironment is characterized by poor vascular perfusion and hypoxia [13] may in part explain why in two phase III studies, bevacizumab (an angiogenesis inhibitor) failed to show survival benefit when added to standard of care gemcitabine [15] or gemcitabine plus erlotinib [16]. More recently trials have begun to evaluate agents with the potential to selectively target this hypoxic microenvironment. A recently published randomized phase II trial evaluated gemcitabine with or without evofosfamide, a chemotherapy prodrug that is selectively activated under hypoxic conditions, in patients with locally advanced and metastatic pancreatic cancer (NCT01144455). Two hundred fourteen patients were enrolled, 77 % of which had metastatic disease. Median PFS was significantly longer for those receiving evofosfamide in addition to gemcitabine (5.6 vs. 3.6 months; HR 0.61; 95 % CI 0.43 to 0.87;  $P = 0.005$ ) [17].

## Overcoming local immunosuppression

Growing evidence suggests that the highly immunosuppressive microenvironment of these tumors also contributes greatly to patient prognosis. Hiraoka et al. conducted a retrospective analysis of 198 patients with pancreatic ductal adenocarcinoma (PDA), 51 patients with intraductal papillary mucinous neoplasms, and 15 patients with nonneoplastic pancreatic lesions who had undergone surgical resection. They found that the levels of Tregs were significantly higher in tumors as compared to nonneoplastic pancreatic lesions ( $P < 0.0001$ ). Lower prevalence of Tregs in the tumor microenvironment was independently ( $P < 0.0001$ ) associated with better survival. In addition, CD8<sup>+</sup>TIA-1<sup>+</sup> cytotoxic T-cell infiltration in pancreatic ducts significantly decreased with progression from low-to high-grade disease, while Treg infiltration increased significantly with progression of tumor grade [18]. Kurahara et al. evaluated 76 patients with pancreatic cancer who underwent surgical resection and found that higher levels of CD163<sup>+</sup> and CD204<sup>+</sup> M2 polarized tumor-

associated macrophages (TAM) in tumor specimen were associated with significantly accelerated lymph node metastasis ( $P = 0.0175$ ) and overall poorer survival ( $P = 0.0171$ ) [19]. In another study evaluating 40 patients with pancreatic cancer who had undergone tumor resection, Shibuya et al. found that CD11b+ myeloid-derived suppressor cells (MDSC) were significantly increased in poorly differentiated tumors as compared to well to moderately differentiated tumors ( $P = 0.04$ ) [20]. In addition, they also found that the majority of intratumoral T-cells expressed programmed death-1 (PD-1), a known co-inhibitory receptor of T-cells, which when bound to its ligand PDL1 results in T-cell tolerance and immune evasion. In a separate study, Geng et al. looked at 40 human pancreatic cancer samples along with eight normal pancreatic specimens and found that PDL1 expression was significantly greater on pancreatic carcinoma specimens compared with normal pancreatic tissue ( $P < 0.01$ ). In addition, PDL1 expression was significantly associated with poor tumor differentiation ( $P < 0.01$ ) and advanced tumor stage ( $P < 0.01$ ) [21].

Given the growing data on the immunosuppressive microenvironment of these tumors and its correlation with poorer outcomes, efforts are being made to overcome this immune evasion and develop effective anti-tumor immune responses. One method being studied is the inhibition of known immunosuppressive cytokines in the tumor microenvironment. TGF- $\beta$ 2 is such a cytokine, which has also been shown to have tumorigenic qualities [22]. As a broadly immunosuppressive cytokine, TGF $\beta$  down-regulates activated cytotoxic T-cells and natural killer (NK) cells and induces the differentiation of naive CD4+ T-cells to regulatory T-cells (Tregs) [23]. Blocking TGF- $\beta$ 2 with trabedersen, an antisense oligonucleotide against TGF- $\beta$ 2 mRNA, has shown encouraging results in a phase I/II study including a complete response of liver metastases in one patient with metastatic pancreatic cancer who is still alive after 75 months [24]. Another approach involves generating a macrophage-dependent immune response against the tumor. A phase I study evaluating CP-870,893, an agonist of CD40 (a costimulatory protein for macrophage cells) in combination with gemcitabine in patients with metastatic pancreatic cancer, has also shown promising results including 2 out of 21 patients with complete responses of liver metastases and an improved PFS and OS (5.6 and 7.4 months respectively) as compared to historical controls with gemcitabine monotherapy (2.3 and 5.7 months, respectively) [25].

A third method is researching the removal of immune checkpoints known to block T-cell-dependent anti-tumor immune responses. Unfortunately, trials evaluating immune checkpoint inhibitors as monotherapy in this disease have to date been unsuccessful. In a multicenter phase I trial of anti-PDL-1 in 17 patients with advanced pancreatic cancer, no responses were found [26]. In addition, a phase II trial of single-agent ipilimumab in 27 patients with locally advanced or metastatic pancreatic cancer also showed no responses [27]. One of the obstacles with using single-agent immunotherapy in metastatic pancreatic cancer may be the limited amount of time these therapies have to stimulate immune responses given these patients' overall poor prognosis. In clinical trials evaluating vaccine therapies and immune checkpoint inhibitors in other solid cancers including prostate cancer and melanoma, the time until clinical benefit with therapy (time to separation of OS curves between therapy and control) ranged from 4–5 months to 14–15 months [28–31]. Even for anti-PD1 and anti-PDL1 therapies, specifically, which seem to work in a shorter timeframe,

these agents may have difficulty inducing effective anti-tumor immune responses given the mean survival for patients with metastatic pancreatic cancer receiving second-line therapy may be less than 3 months [32]. To avoid this issue, these immunotherapies are being combined with standard chemotherapy in the first line and adjuvant settings where survival times are longer. For example, a phase I trial is evaluating ipilimumab in combination with gemcitabine in locally advanced or metastatic disease (NCT01473940) and a phase II trial is evaluating CT -Oil, an anti-PD-1 antibody, in combination with gemcitabine in the adjuvant setting (NCT01313416). In preclinical work, gemcitabine has also been found to selectively deplete MDSCs [33, 34], suggesting that it may have a role as an immune enhancer when combined with immunotherapies. Another approach which is being researched is the combination of immune checkpoint inhibitors with other immune augmenting agents to help enhance the anti-tumor immune response. In preclinical studies, radiation has been found to enhance anti-tumor immune responses by making tumors more immunogenic and sensitizing them to cytotoxic T-cell killing [35–37]. Based upon these findings, a phase I trial is evaluating the combination of MEDI4736, an anti-PDL1 agent and/or tremelimumab, a CTLA-4 inhibitor, with stereotactic body radiation therapy in patients with unresectable pancreatic cancer [NCT02311361]. In addition, preclinical work in a murine model of pancreatic cancer, which initially found no response to anti-PDL1 monotherapy, has found that the chemokine CXCL12 produced by a subgroup of stromal fibroblasts, serves an immunosuppressive role in the tumor microenvironment and blocking its receptor CXCR4, with AMD3100 dramatically enhances the anti-tumor response of anti-PDL1 in a T-cell-dependent manner [38]. In line with this work, a phase 1 trial is evaluating AMD3100 in advanced pancreatic as well as ovarian and colorectal cancer [NCT02179970].

## Eliciting immune responses

Another strategy being evaluated in the clinic is vaccine therapy, which primes the immune system with antigens expressed on the tumor to stimulate de novo immune responses. A comprehensive review of the potential tumor-specific antigens as well as vaccine strategies being evaluated in pancreatic cancer is beyond the scope of this article, but it can be found in separate reviews [39]. Still perhaps the most well-studied vaccine platforms are GVAX pancreas and CRS-207.

GVAX pancreas consists of two irradiated allogeneic pancreatic tumor cell lines, which have been engineered to express granulocyte-macrophage colony-stimulating factor (GM-CSF) and have been shown in early-phase studies to induce T-cell responses to tumor antigens such as mesothelin [40]. CRS-207 is a live attenuated *Listeria monocytogenes*, which has been engineered to express mesothelin. Early-phase clinical trials have shown that this vaccine can induce anti-tumor immune responses [41]. In a recently published trial, Le et al. randomized 90 patients with metastatic pancreatic cancer 2:1 to two doses of low-dose cyclophosphamide (given to inhibit Tregs) together with GVAX (given as an immune primer) followed by four doses of CRS-207 (given as an immune boost) or six doses of low-dose cyclophosphamide/GVAX every 3 weeks [42]. 97 % of patients had received prior chemotherapy. Overall survival (OS) was significantly longer for those receiving GVAX with CRS-207 boost compared to those receiving GVAX alone (6.1 vs. 3.9 months; HR 0.59;  $P = 0.02$ ). Furthermore, CD8 T-cell responses against mesothelin were found to be

associated with longer OS ( $P = 0.0033$ ). The survival benefit seen with GVAX/CRS-207 is on par with historical controls receiving second-line chemotherapy for metastatic disease [32]. Although this treatment seems to offer only modest survival benefit, there is hope that its combination with anti-PD-1 therapy in ongoing clinical trials [NCT02243371] will further improve antitumor immune responses and overall outcomes. In a phase Ib trial, which randomized 30 patients with previously treated advanced pancreatic cancer to ipilimumab 10 mg/kg with or without GVAX vaccine, the median overall survival analysis favored the combination arm as compared to ipilimumab alone (5.7 vs. 3.6 mo, HR 0.51,  $P = 0.072$ ; 1 year OS 27 vs. 7 %) [43]. In addition, early-phase trials are also evaluating standard chemotherapy (FOLFIR-INOX) in combination with GVAX and ipilimumab [NCT01896869] as well as other pancreatic cancer vaccines in combination with anti CTLA-4 therapy [NCT00836407].

### Targeting therapy to the immunosuppressed microenvironment

In addition to immunotherapies, there may still be other novel ways to target the microenvironment of pancreatic cancer. Instead of trying to overcome the immunosuppressive environment of these tumors, one approach may be to use cytotoxic therapies designed to specifically localize to these immunosuppressed areas.

In preclinical work, Kim et al. found that not only could a recombinant attenuated form of *Listeria monocytogenes* elicit an anti-tumor immune response by presenting tumor-associated antigens (TAA) to antigen presenting cells (APCs), but in mouse models of breast cancer these bacteria could also localize to and infect tumor cells [44]. In addition, they found that these attenuated listeria were efficiently removed by a healthy immune response in normal tissues by 3–5 days, yet continued to accumulate in the immunosuppressive microenvironments of tumors. Building off these results, Quispe-Tintaya hypothesized that these attenuated recombinant listeria could be used to effectively deliver cytotoxic therapy specifically to the immunosuppressive microenvironments of tumors. To test this hypothesis, they evaluated a radioisotope with cytotoxic ability,  $^{188}\text{rhenium}$ , bound to attenuated live listeria in a mouse model of metastatic pancreatic cancer [45]. They first looked at the attenuated listeria alone. Similar to the previous study, they found these attenuated listeria were able to multiply effectively in the immunosuppressive microenvironments of metastases and to a lesser extent in the primary tumor but not in normal tissues. The number of listeria peaked in metastases 3 days after administration and after 7 days listeria had been cleared from healthy tissues and could only be found in the primary tumor and metastases. To evaluate  $^{188}\text{rhenium}$  labeled attenuated listeria, they randomized 60 mice to four groups including intraperitoneal injections of  $^{188}\text{rhenium}$ -labeled listeria, listeria alone,  $^{188}\text{rhenium}$  alone, or saline. These treatments were started on day 3 following the injection of pancreatic cancer cells into the mammary fat pad and were given on days 3–9 and then on days 16–19 (estimated cumulative dose of 30 Gy). As compared to those receiving  $^{188}\text{Re}$  or saline alone, mice receiving radiolabeled listeria experienced an average 90 % reduction in the weight of metastasis after 21 days and an average 64 % reduction in the weight of the primary tumor. Although not nearly to the same degree, those mice receiving listeria alone also experienced tumor reduction (50 % reduction in metastases and a 20 % reduction in the primary tumor). These findings further suggest that these attenuated listeria alone may have anti-tumor



activity and that the addition of a cytotoxic entity to these listeria may significantly enhance these responses.

Interestingly enough, expecting that responses to listeria alone where due to an immune response against infected tumor cells, Quispe-Tintaya et al. measured anti-listeria-specific CD8 T-cell responses and found that the responses were weak and did not differ significantly from the saline-treated groups. These data are understandable since one would not expect a new immune response to be acquired in less than 3 weeks. In other preclinical work, attenuated listeria has been shown to have immunogenic-independent anti-tumor activity related to generation of high levels of reactive oxygen species inside tumor cells [44, 45]. Still despite these findings in mouse models, this does not preclude the possibility that attenuated listeria-based vaccines may also help prime or boost anti-tumor immune response in humans when given enough time.

In theory, radioactive attenuated listeria offers a number of benefits as a possible treatment for pancreatic and other solid cancers. First, as just discussed, this mode of therapy does not require the months necessary for an anti-tumor immune response to develop, a luxury which many patients with metastatic pancreatic cancer may not have. Second, unlike most immunotherapies, it is not subject to the dozens of mechanisms tumors use to evade immune responses (PD1/PDL1 or CD80/CTLA-4 checkpoint blockade, lack of HLA expression on tumor cells, presence of Tregs, TAMs, MDSCs, or immunosuppressive agents such as indoleamine 2,3-dioxygenase and TGFβ in tumor microenvironments) [46]. However, as with all radiotherapy, it is limited by the maximum cumulative dose that can be received before life-threatening toxicities develop. This characteristic severely limits the opportunity for repeated treatments even if this therapy proves effective in clinical trials.

## Nanoparticles as novel therapy

However, there may still be other novel cytotoxic agents that evade immunogenic blockade and at the same time are not subject to multidrug-resistant clones or curtailed by maximum lifetime doses. Plasmonic nanoparticles are particles that can generate heat in response to the application of non-ionizing electromagnetic radiation. This heat is usually generated through the excitation and relaxation of electrons [47]. Iron-oxide-based nanoparticles are magnetically sensitive nanoparticles, which have an ability to generate heat when an oscillating magnetic field is applied [48]. Similar iron-oxide-based nanoparticles, such as Gastromark, have been FDA approved as MRI contrast agents, although doses used for diagnostic purposes are usually less. Matthew et al. evaluated an iron-oxide-based nanoparticle in combination with an alternating magnetic field (AMF) in a murine model of pancreatic cancer [49]. They found that mice receiving this therapy lived 31 % longer than mice in the control groups ( $P < 0.005$ ). In a separate murine model of pancreatic cancer, Kossatz et al. demonstrated that tumor growth was severely reduced following treatment with iron-oxide-based nanoparticles and AMF application. The tumor volumes in treated mice were 50–60 % smaller 28 days after initiation of therapy as compared to mice in the untreated group [50]. Perhaps the most dramatic results investigating this approach in murine models of pancreatic cancer were reported by Wang et al. [51]. In this study, 98 mice containing pancreatic tumor xenografts were treated in four groups including no treatment,

AMF alone, direct injection of iron-oxide-based magnetic fluid, or the combination of directly injected iron-oxide-based magnetic fluid followed 24 h later by application of AMF (300 kHz, 110 Gs). After application of AMF for 30 min, tumors in the combination arm reached temperatures in the range of 47–51 °C, well above tumoricidal temperatures, while core body temperature remained within normal range. All 20 mice in this group had complete responses to therapy. Of these, five mice had relapsed disease within 2 months, while the other 15 mice were free of disease and still alive after 140 days of followup. Survival seen in this group was significantly better than all three control groups where all mice were dead by 63 days of follow-up ( $P < 0.05$ ).

These findings are promising and suggest that magnetic nanoparticles in combination with AMF may yet have a role in pancreatic cancer treatment. One hurdle of such therapy has been selectively targeting these agents to tumors. In preclinical work with other solid tumors, magnetic nanoparticles administered intravenously without any additional directing agents were able to effectively localize to tumors producing dramatic results [52]. In this study, it was hypothesized that nanoparticles were able to localize to tumors due to their selectively leaky vasculature. Yet these results have not been replicated and it is unclear if similar responses would be found in pancreatic cancer, which has been shown to have a highly desmoplastic and fibrotic microenvironment characterized by poor vascular patency and perfusion [13]. One approach to overcome this potential obstacle may be to link these nanoparticles to other tumor-directing agents such as attenuated listeria [44, 45].

## Conclusion

In conclusion, historically patients diagnosed with metastatic pancreatic cancer have faced a grim prognosis. Evidence suggests that the microenvironment of pancreatic cancer may contribute greatly to this prognosis. This microenvironment has a dense fibrotic stroma, and is hypoxic and highly immunosuppressive, all of which pose barriers to treatment. New stromal disrupting agents, hypoxia-activated chemotherapies, and immune-based therapies are currently undergoing clinical evaluation and offer promise. In addition to these therapies, preclinical work evaluating novel cytotoxic agents including nanoparticles is also encouraging. While much research still needs to be done, these strategies offer new hope for patients with pancreatic cancer.

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